Decreased Hypersensitivity to Xanthomonads in Pepper after Inoculations with Virulent Cells of Xanthomonas vesicatoria

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ABSTRACT

Living cells of a virulent strain of *Xanthomonas vesicatoria* decreased hypersensitivity in pepper leaf tissue to an avirulent strain of the organism. Moreover, reduced hypersensitivity occurred only when cells of the virulent strain were injected into pepper leaves prior to injections with the avirulent cells. The attenuation of hypersensitivity increased with time between inoculations. The virulent cells of *X. vesicatoria* decreased hypersensitivity initiated by some other xanthomonads, but not by some species of *Pseudomonas*. Heat-killed cells of *X. vesicatoria* were also effective in reducing hypersensitivity initiated by avirulent cells of the same organism. It was theorized that a zone of influence was exerted on the host cells by the virulent bacteria and that the zone increased with time of incubation. Hypersensitivity initiated by avirulent cells was prevented, if avirulent cells were deposited in the zone of influence, but not if they were deposited elsewhere on the cell surface.

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Additional key words: induced susceptibility.

The hypersensitive interaction (HR) is important in limiting pathogenicity of many organisms (4, 7). It is a factor that must be considered in pathogenesis of certain races of *Xanthomonas vesicatoria* (Doidge) Dows. to certain cultivars of pepper (*Capsicum annuum* L.). If any plant-parasitic bacterium could prevent HR in its host, then the bacterium might multiply to populations that initiate disease. Experiments designed to study the effectiveness of a virulent bacterium in blocking HR in pepper are reported here.

MATERIALS AND METHODS.—Only one cultivar of pepper, 23-1-7-BK, was used in this work. Plants of this cultivar have in the homozygous condition a dominant gene for hypersensitivity to certain strains of *X. vesicatoria* (1). Fully expanded, but not senescent, leaves were inoculated, after which plants were placed in a growth chamber at 30 C with a 14-h light period. Twenty-five centimeters from the lights, ca. 6,804 lux (630 ft-c) were delivered.

Two strains of *X. vesicatoria* were used; one, E-3, (avirulent), initiated hypersensitivity in 23-1-7-BK and the other, 68-1, (virulent), initiated susceptibility. The avirulent strain was pathogenic to plants of the pepper cultivar, Early Calwonder. Other xanthomonads and pseudomonads used in the tests were isolated from diseased plants, maintained in lyophilized cultures, and were pathogenic to their respective hosts. The bacteria were cultured in nutrient broth for 24 h and centrifuged from the medium. They were resuspended in sterile distilled H_2O and the numbers adjusted to 10^8 cells/ml by standardizing turbidity as measured with a Spectronic 20. Other inocula were obtained by appropriate dilutions, or concns, from the standard. Heat-killed bacteria were prepared by heating suspensions to 60 C for 1 h in a water-bath. This was done after adjusting numbers of bacteria. Leaves were inoculated by injection-infiltration of the intercellular areas with a hypodermic syringe.

Electrolytes.—Cellular damage was assessed in terms of electrolyte leakage from leaf tissues. Leakage from leaf disks submerged in distilled H_2O was measured with a YSI Model 31 conductivity bridge with a 0.1 cell constant. Leaf disks were cut with a cork borer (1.5-cm diam) and either 7, or 15 disks, (depending upon experiments), were placed in a 50-ml beaker containing 20 ml of distilled H_2O. The disks were kept submerged with a plastic screen and conductivity values were obtained. Immediately after the first reading, the leaf disks in the H_2O were placed under vacuum of 635 mm (25 inches) of Hg for 2 min and were infiltrated upon release of the vacuum. The disks were shaken at 200 oscillations per min until the second reading was taken 1 h later. The difference in readings represented leakage/hour.

All treatments were replicated three times, in each experiment, and experiments were repeated at least three times. Data were analyzed statistically by a pair-difference test (6).

RESULTS.—*Mixtures of virulent and avirulent living cells.*—Mixtures of virulent and avirulent cells of *X. vesicatoria* were injected into pepper leaves and the resulting amounts of electrolyte leakage were compared with similar treatments in which virulent and avirulent cells were used alone. Three concns of avirulent cells and one concn of virulent cells were used in all tests.

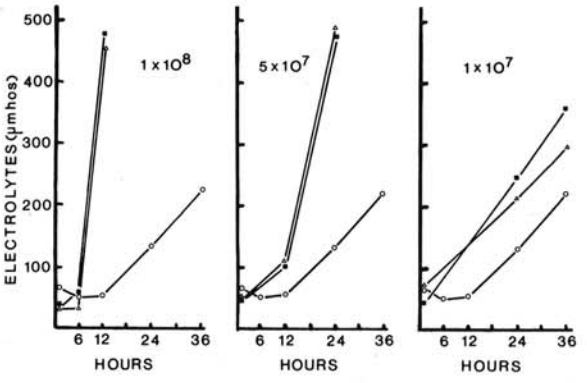

Fig. 1. Rates of electrolyte leakage from pepper leaf tissue inoculated with [O] avirulent; [■] virulent; and [△] mixtures of avirulent and virulent cells of *Xanthomonas vesicatoria*. The concn of virulent cells was held constant at 10^8 cells/ml, but concns of avirulent cells were varied and indicated on the graph.

TABLE 1. Electrolyte leakage from pepper leaf tissue initiated by an avirulent strain of *Xanthomonas vesicatoria* injected into the leaves at various time intervals following injection of virulent cells of the same organism into half of each leaf

Incubation period	Electrolyte leakage			
	Avirulent[a]	Virulent+ avirulent[a]	Reduction	
(h)	(μmhos)	(μmhos)	(μmhos)	(%)
0	308	367	−59*[b]	0
3	408	339	69	14
6	360	248	112**	31
9	266	82	184**	68
12	466	105	361**	77

[a]Determinations were made from leaves in which half of each were inoculated with virulent cells and the whole leaf was inoculated with avirulent cells. Leakage values were obtained from each half-leaf and designated avirulent if the half only received avirulent cells and virulent-avirulent if the half received both kinds of bacteria. Values are average of nine determinations.

[b]* = Significant reduction, $P = 0.05$; ** = significant reduction, $P = 0.01$.

TABLE 2. Electrolyte leakage from pepper leaf tissue initiated by an avirulent strain of *Xanthomonas vesicatoria* injected into the leaves following injection of various concns of virulent cells of the same organism into half of each leaf

Concn virulent	Electrolyte leakage			
	Avirulent[a]	Virulent+ avirulent[a]	Reduction	
(cells/ml)	(μmhos)	(μmhos)	(μmhos)	(%)
10^9	515	322	130**[b]	37
10^8	528	413	115*	22
10^7	479	542	−63**	0
10^6	602	693	−91**	0

[a]Determinations were made from leaves in which half of each were inoculated with virulent cells and the whole leaf was inoculated with avirulent cells. Leakage values were obtained from each half-leaf and designated avirulent if the half only received avirulent cells and virulent-avirulent if the half received both kinds of bacteria. Values are average of nine determinations.

[b]* = Significant reduction, $P = 0.05$; ** = significant reduction, $P = 0.01$.

The lowest concn (10^7 cells/ml) of avirulent cells initiated a more rapid rate of electrolyte leakage from leaf tissue than that initiated by the virulent cells in 10 times greater concns (Fig. 1). Electrolytes leaked from tissues injected simultaneously with avirulent and virulent cells about as rapidly as they did from tissues treated with avirulent cells alone. When in mixtures, therefore, the effects of the avirulent cells dominated over the effects of the virulent cells. This was true, even when the concns of virulent cells in the mixtures were 10 times that of avirulent cells.

Virulent cells preceding avirulent cells.—The dominance of HR was further studied in tests in which living virulent cells were injected into leaves at several time intervals preceding avirulent cells. A suspension of virulent bacteria in concns of 10^8 cells/ml were injected into half-leaves of pepper at 0, 3, 6, 9, and 12 h preceding injections of avirulent cells at the same into the whole leaves. Controls were leaves treated similarly but injected with sterilized distilled-water instead of avirulent bacteria. Electrolyte leakage from both halves of leaves was determined 12 h after injection of the avirulent cells.

The mixture of the two kinds of cells injected simultaneously (0 h) resulted in a significant increase in loss of electrolytes over that caused by the avirulent cells alone (Table 1). Using this as a base, the presence of virulent cells in host tissue for 3 h affected the rate of leakage of electrolytes caused by avirulent cells.

The longest interval, 12 h, between inoculations of virulent and avirulent cells had the greatest effect. But even then, complete protection from the effects of the avirulent cells was not afforded by the virulent cells; greater leakage occurred than with controls which consisted of virulent cells alone. Nevertheless, the virulent bacterial cells prevented some leakage of electrolytes initiated by avirulent bacteria.

Concentrations of virulent cells in primary inoculation.—The concns of virulent cells in the first inoculation were varied in some experiments. Inocula containing 10^9, 10^8, 10^7, and 10^6 cells/ml of the virulent isolate were injected into half-leaves of pepper. Avirulent bacteria at 10^8 cells/ml were injected into the whole leaf 12 h later. Controls consisted of injecting sterilized distilled water into the whole leaf in place of avirulent bacteria.

Inocula of at least 10^8 cells/ml of virulent cells had been used to reduce the dominance of the HR (Table 2). Virulent cells might have reduced electrolyte leakage even further if the interval between inoculations had been increased. In other experiments, inocula of 10^5 cells/ml of virulent bacteria were injected into half-leaves and followed by avirulent cells at concns of 10^8 cells/ml for up to 120 h after the primary inoculation. In no instance, did the virulent cells at low concns reduce electrolyte leakage incited by the avirulent cells injected afterward.

Concentration of avirulent cells in secondary inoculation.—To study the magnitude of the effect of the virulent bacteria on host cells, the concns of avirulent bacterial cells in the secondary inoculations were varied. Twelve hours after injecting inoculum containing 10^8 cells/ml of virulent bacteria into half-leaves of pepper, concns of 2×10^8, 1×10^8, and 0.5×10^8 cells/ml of avirulent cells were injected into the whole leaves. The control consisted of injecting sterilized distilled water into whole leaves instead of avirulent bacteria.

The virulent bacteria decreased electrolyte leakage with all concns of avirulent cells in the secondary inoculations (Table 3). Though the effect was greatest with the lowest concn of avirulent cells, a trend toward a lesser effect was noted as the concn of avirulent cells increased. For experimental purposes, then, the greatest reduction in HR would probably occur at the concn of avirulent cells in the secondary inoculation that consistently initiates confluent HR in the leaf tissue.

Other bacterial combinations.—The effect of virulent cells of *X. vesicatoria* in decreasing HR by some other xanthomonads and pseudomonads was observed. The

bacteria used (Table 4) also initiated HR in pepper. As in other experiments, the virulent cells (10^8 cells/ml) were first injected into half-leaves of pepper, and 12 h later the various bacteria at 10^8 cells/ml were injected into the whole leaves. Controls were injected with sterilized distilled water instead of bacteria in the second inoculation.

Virulent cells of *X. vesicatoria* decreased the leakage of electrolytes incited by other xanthomonads, but not that initiated by the pseudomonads tested. Some sort of specificity seemed to exist in the reduction of leakage by virulent cells of *X. vesicatoria*.

Heat-killed virulent cells in primary inoculation.—Heat-killed cells of *X. vesicatoria* were substituted for living virulent bacteria in the first inoculation in some experiments. Abscission of leaves often occurred within 60 h after the introduction of heat-killed cells. To determine whether this abscission was associated with cell necrosis, electrolyte leakage determinations were made periodically over a 48-h interval after leaves had been injected with heat-killed cells at concns of 10^9 cells/ml. No significant increase of electrolyte leakage occurred, although there was a trend toward increased leakage with time.

Reduction of electrolyte leakage occurred if heat-killed bacterial cells were used in the first inoculation and followed by living avirulent cells 24 h later in a second inoculation. Electrolyte leakage was determined 8 h after the second inoculation. This effect was not noted when mixtures of heat-killed virulent and living avirulent cells were injected simultaneously.

A concn of 10^9 cells/ml of heat-killed bacteria was necessary for significant reduction of electrolyte leakage from tissue that was subsequently inoculated with avirulent bacteria at a concn of 10^8 cells/ml (Table 5). Considerable variation occurred in the tests using heat-killed bacteria and large differences were necessary for significance.

In other experiments, a concn of 10^9 cells/ml of heat-killed bacteria was used in first inoculations and 5×10^7 cells/ml of living avirulent bacteria were used 24 h later in the second inoculations. Both heat-killed virulent and heat-killed avirulent bacteria reduced the amounts of electrolyte leakage 8 h after the second inoculations. Likewise, heat-killed cells of *P. solanacearum* could be substituted for heat-killed cells of *X. vesicatoria*. Therefore, no specificity in the prevention of HR was noted among heat-killed bacteria.

DISCUSSION.—The spatial relationship of the bacterial cells on the surface of host cells is an important consideration in interpretation of the effect of virulent cells on hypersensitivity caused by avirulent cells in subsequent inoculations. Ercolani (3) proposed that bacteria become attached to multiplication sites on the cell surface, and, depending upon the factors contained by the bacterium, a susceptible, hypersensitive, saprophytic, or protective effect is produced. If all such sites were occupied by virulent bacteria in the first inoculation, then no sites would be available for avirulent cells in subsequent inoculations. With inoculum of 10^8 cells/ml in the first inoculation however, some multiplication sites would probably still be available. Counts of cells in pepper leaf tissue represented by 1 mm^2 of leaf surface, and the calculations of bacteria in the volume of inoculum needed to flood the intercellular spaces of that area of leaf tissue, place the number of bacterial cells per host cell on an approximate one to one basis. Therefore, unless only one multiplication site exists per cell, not all sites would be covered after the first inoculation.

The effect on hypersensitivity can be explained by an hypothesis alternate to that of Ercolani (3). In the alternate hypothesis, portions of the host cells are influenced by the virulent bacterium in the first inoculum. This "zone of influence" increases with time after deposition on the cell wall. If an avirulent bacterium was deposited in the zone influenced by the virulent cell, no effect on the cell would occur. But, typical

TABLE 3. Electrolyte leakage from pepper leaf tissue initiated by various concns of avirulent cells of *Xanthomonas vesicatoria* following injection of virulent cells of the same organism into half of each leaf

Concn avirulent (cells/ml)	Electrolyte leakage: Avirulent[a] (μmhos)	Virulent+ avirulent[a] (μmhos)	Reduction (μmhos)	Reduction (%)
2×10^8	537	319	218**[b]	41
1×10^8	529	205	324**	61
5×10^7	643	215	428**	67
0	44	75	−31**	0

[a]Determinations were made from leaves in which half of each were inoculated with virulent cells and the whole leaf was inoculated with avirulent cells. Leakage values were obtained from each half-leaf and designated avirulent if the half only received avirulent cells and virulent-avirulent if the half received both kinds of bacteria. Values are average of nine determinations.

[b]** = significant reduction, $P = 0.01$.

TABLE 4. Electrolyte leakage from pepper leaf tissue initiated by isolates of various *Xanthomonas* and *Pseudomonas* spp. following injection of virulent cells of *X. vesicatoria* into half of each leaf

Avirulent bacterium	Electrolyte leakage: Avirulent[a] (μmhos)	Virulent+ avirulent[a] (μmhos)	Reduction (μmhos)	Reduction (%)
X. vesicatoria	285	50	206**[b]	73
X. campestris	258	65	193**	71
X. phaseoli	195	87	108**	55
P. tabaci	207	254	−47	0
P. lachrymans	211	284	−73*	0
P. solanacearum	195	259	−64**	0
H_2O	21	48	−27**	0

[a]Determinations were made from leaves in which half of each were inoculated with virulent cells and the whole leaf was inoculated with avirulent cells. Leakage values were obtained from each half-leaf and designated avirulent if the half only received avirulent cells and virulent-avirulent if the half received both kinds of bacteria. Values are average of nine determinations.

[b]* = Significant reduction. $P = 0.05$; ** = significant reduction, $P = 0.01$.

TABLE 5. Electrolyte leakage from pepper leaf tissue initiated by avirulent cells of *Xanthomonas vesicatoria* injected into the leaves following injection of various concentrations of heat-killed cells of the same organism into half of each leaf

	Electrolyte leakage					
Concn heat-killed cells (cells/ml)	Avirulent[a] (μmhos)	Heat-killed cells+ avirulent[a] (μmhos)	Reduction (μmhos)	H_2O (μmhos)	Heat-killed cells+ H_2O (μmhos)	Reduction (μmhos)
H_2O	454	359	−95	41	52	−11
10^7	468	344	−124	51	67	−16
10^8	531	356	−175	47	56	−9
10^9	515	132	−383**[b]	42	54	−12

[a]Determinations were made from leaves in which half of each were inoculated with virulent cells and the whole leaf was inoculated with avirulent cells. Leakage values were obtained from each half-leaf and designated avirulent if the half only received avirulent cells and virulent-avirulent if the half received both kinds of bacteria. Values are average of nine determinations.
[b]** = significant reduction, $P = 0.01$.

hypersensitivity would occur if an avirulent bacterium was deposited outside that zone of influence.

For example, when virulent and avirulent cells were mixed and introduced simultaneously, the influence of the virulent cells on the host cell was very small. However, the zone of influence increased within 12 h after inoculation with the virulent bacteria. The percentage reduction in electrolyte leakage in the experiments reported herein, reflected the percentage of each cell influenced by the virulent bacterium, since only those avirulent cells deposited on "noninfluenced" zones of each cell would initiate hypersensitivity and therefore, leakage of electrolytes. Consequently, if 50% of a cell was influenced by a virulent bacterium, only 50% of the subsequently inoculated avirulent bacteria would be deposited on noninfluenced zones. A 50% reduction in electrolyte leakage would be expected.

The zone of influence increased at a rate greater than can be accounted for by multiplication of bacteria. After 12 h, the zone of influence was usually more than 50% of the cell. The generation time for *X. vesicatoria* in pepper leaf tissue is ca. 8 h (10). Therefore, only slightly more than one generation would be expected in the 12-h period.

Daley (2) introduced the concept of "induced susceptibility" and pointed out that it is just as compatible with published data as the concept of "induced resistance." Although he worked with an obligate parasite, *Puccinia graminis tritici*, Daley wrote, "there may be a general, but perhaps incorrect, tendency to relegate the notion only to cases of obligate parasitism." Our data may extend the concept of induced susceptibility to facultative parasitism.

In Daley's work with the rust, the mechanism of induced susceptibility was reversion from hypersensitivity to susceptibility. Complete reversion to susceptibility was not achieved in pepper, but interference of HR by virulent cells of *X. vesicatoria* was clearly demonstrated. With proper conditions complete reversion may be possible.

Superficially, the factor in living bacteria that prevented HR appeared to be relatively heat-stable, since heat-killed bacteria also prevented HR. Heat-killed cells were not as effective, however, as living cells, because a greater concn of heat-killed cells was required and more variation within experiments was evident when using heat-killed cells instead of living virulent bacteria. Ercolani (3) had similar results with *Corynebacterium michiganense* in tomato.

Lozano and Sequeira (5) found that heat-killed cells of *P. solanacearum* prevented HR in tobacco leaf tissue. They attributed the effect of the heat-killed cells to the induction of nonspecific inhibitory substances in tobacco leaf tissue that prevented establishment of avirulent cells in the challenge inoculation. The effect of the living virulent cells in this work could not have been similar, because the virulent cells multiply in the host leaf. Formation of inhibitory substances would prevent establishment of the virulent bacteria, also. There is evidence, then, that heat-killed and living virulent bacteria could prevent HR by different mechanisms.

Novacky et al. (8) reported that low numbers of living avirulent bacteria prevented HR in tobacco initiated by higher numbers of the same bacterium inoculated later. They pointed out that HR can be prevented by other diverse treatments. Whether or not the mechanism of prevention of HR by living virulent bacteria is similar to that of other treatments giving the end-result must await further research.

The HR initiated in pepper by species of *Pseudomonas* was not affected by the preceding inoculations with living virulent cells of *X. vesicatoria*. This suggests that the HR initiated by isolates of *Xanthomonas* in pepper was not identical to that initiated by the pseudomonads. Thus, the problem of hypothesizing the universality of the HR has been emphasized.

Our data demonstrate a very early effect of virulent bacteria on the host cells. A significant effect was noted within 6 h of deposition of the bacteria on the cell surfaces. Histological evidence of an effect was not noted before 24 h after inoculations of virulent bacteria, but changes were noted in the endoplasmic reticulum by 36 h (9). Similarly, electrolyte leakage does not increase significantly within 24 h of the introduction of virulent bacteria into leaf tissues; it then increases gradually through 72 h. Therefore, effects of virulent bacteria on host cells were noted earlier than expected on the basis of previous work.

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